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(54) Title: METHODS TO INCREASE BLOOD FLOW TO ISCHEMIC TISSUE			
(57) Abstract The present invention provides methods and kits for increasing blood flow to ischemic tissue, comprising the administration of an effective amount of angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, angiotensin II (AII), AII analogues, AII fragments or analogues thereof or AII AT ₂ type 2 receptor agonists.			

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METHODS TO INCREASE BLOOD FLOW TO ISCHEMIC TISSUE

Cross Reference

5 This application is a Continuation-In-Part of U.S. Provisional Application Nos. 60/096,414 filed August 13, 1998 and 60/101,024 filed Sept. 18, 1998.

Background of the Invention

Collateral circulation to tissues can be stimulated in two independent ways: 1) the
10 formation of new blood vessels through angiogenesis and 2) the enhancement in function of preexisting blood vessels (Fernandez et al., *J. Lab. Clin. Med.* 105:141-145 (1985)).

Angiogenesis is the process by which new blood vessels are formed from existing capillaries. (U.S. Patent No. 5,318,957, incorporated by reference herein in its entirety.) Angiogenesis plays an important role in such widely divergent biological conditions as
15 embryonic development, tumor growth, wound healing, and chronic inflammatory disease. (Folkman et al., *Science* 235:442-447 (1987))

Capillaries are composed almost entirely of endothelial cells. Angiogenesis comprises a cascade of events, including protease secretion by endothelial cells, degradation of the basement membrane, migration through the surrounding matrix,
20 proliferation, alignment, differentiation into tube-like structures, and synthesis of a new basement membrane. (U.S. Patent No. 5,318,957)

Several angiogenic agents with different properties and mechanisms of action are well known in the art, including acidic and basic fibroblast growth factor, transforming growth factor alpha and beta, tumor necrosis factor, platelet-derived growth factor,
25 vascular endothelial growth factor, angiogenin, and haptoglobin. (U.S. Patent No.

5,318,957) However, the therapeutic applicability of some of these compounds is limited by their potent pleiotropic effects on various cell types. Thus, there remains a need in the art for angiogenic agents with more general applicability.

Very few therapies have been demonstrated to increase collateral circulation by enhancing the function of existing blood vessels. In an isolated case, U.S. Pat. No. 4,296,100 discloses animal experiments wherein bovine fibroblast growth factor (FGF) was injected into the heart to distribute the desired amount of FGF over the area of the heart to be treated. This treatment was given as close to the time the heart attack as possible in order to control damage, possibly by improving collateral circulation, although the exact mechanism is unknown. The experiments showed that a one time treatment could reduce infarct size (area that will scar or remain permanently damaged) in the test animal to one quarter the size of the control (non-treated) hearts. Histological studies did not show a significant increase in capillary areas in the hearts as a result of such treatment with FGF.

15 Congestive heart failure is the leading cause of cardiovascular mortality (Tyagi, *J. Cell Biochem.* 65:388-394. (1997), and is caused by cardiac remodeling that leads to an enlargement of the heart following a myocardial infarction (Pfeffer, *Am. J. Cardiol.* 68:17D-25D (1991)). There is a strong correlation between long term morbidity from congestive heart failure and the degree of post-infarct remodeling. As little as a 35% increase in the volume of the heart in the months following infarction leads to an increase in mortality by a factor of 3-4 (Hammermeister et al., *Circulation* 59:421-435 (1979)). Therefore, therapies that attenuate cardiac remodeling following myocardial infarction are needed to prevent the development of congestive heart disease.

The loss of blood flow to the ischemic tissue is the primary factor in causing cardiac remodeling. Studies show that there is major cardiac remodeling following a myocardial infarction if the infarct-related artery is totally occluded (Kim and Braunwald, *Circulation* 88:2426-2436 (1993)). However, cardiac remodeling can be
5 minimized if blood flow can be restored to the ischemic tissue. In addition, the earlier blood flow is restored the more cardiac remodeling can be attenuated (Hochman et al., *Circulation* 75:299-306 (1987); Bonaduce et al., *J. Am. Coll. Cardiol.* 16:1561-1568 (1990)). Therefore, treatments aimed at quickly restoring blood flow following myocardial infarction can minimize cardiac remodeling and the development of
10 congestive heart disease.

Chemical therapies are currently one of the most useful treatments for restoring blood flow to ischemic tissue following myocardial infarction. Thrombolytic therapies are based on clearing the occluded coronary artery in order to restore blood flow (US
5,589,173). In addition to clearing the occluded coronary artery, the presence of a
15 collateral blood supply can minimize the effect of cardiac remodeling. Therefore, chemical therapies directed at stimulating collateral circulation could minimize cardiac remodeling following myocardial infarction. However, there are currently few materials available for stimulating collateral blood flow.

Based on the above, there remains a need for the development of methods to
20 increase blood flow to ischemic tissue in general, and a particular need for increasing blood flow to the heart following a myocardial infarction.

SUMMARY OF THE INVENTION

The present invention provides improved methods and kits to increase blood flow to ischemic tissue comprising the administration of angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, angiotensin II (AII) analogues, AII fragments or analogues thereof or AII AT₂ type 2 receptor agonists.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a graph showing the effect of AII analogues on blood flow in conjunction with Apligraf treatment.

10 Figure 2 is a graph showing the effect of AII analogues on endothelial cell production in conjunction with Apligraf treatment.

Figure 3 is a graph showing the effect of AII analogues on blood flow after thermal injury.

15 DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

All references, patents and patent applications are hereby incorporated by reference in their entirety.

As used herein, the term "ischemia" refers to any condition associated with an inadequate flow of oxygenated blood to a part of the body. Ischemia occurs any time that blood flow to a tissue is reduced below a critical level. This reduction in blood flow can result from the following non-limiting conditions: (i) the blockage of a vessel by an embolus (blood clot); (ii) the blockage of a vessel due to atherosclerosis; (iii) the breakage of a blood vessel (a bleeding stroke); (iv) the blockage of a blood vessel due to vasoconstriction such as occurs during vasospasms and possibly, during transient

ischemic attacks (TIA) and following subarachnoid hemorrhage. Further conditions in which ischemia occurs, include (i) during myocardial infarction (when the heart stops, the flow of blood to organs is reduced and ischemia results); (ii) trauma; and (iii) during cardiac and neurosurgery (blood flow needs to be reduced or stopped to achieve the aims
5 of surgery).

As used herein, the term "ischemic tissue" refers to any tissue that is receiving an inadequate flow of oxygenated blood.

As used herein, "increasing blood flow" refers to such increases mediated by either the formation of new blood vessels through angiogenesis or the enhancement in
10 function of preexisting blood vessels.

Within this application, unless otherwise stated, the techniques utilized may be found in any of several well-known references such as: *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel,
15 1991. Academic Press, San Diego, CA), "Guide to Protein Purification" in *Methods in Enzymology* (M.P. Deutscher, ed., (1990) Academic Press, Inc.); *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA), *Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed.* (R.I. Freshney. 1987. Liss, Inc. New York, NY), *Gene Transfer and Expression Protocols*, pp. 109-128, ed.
20 E.J. Murray, The Humana Press Inc., Clifton, N.J.), and the Ambion 1998 Catalog (Ambion, Austin, TX).

U.S. Patent No. 5,015,629 to DiZerega (the entire disclosure of which is hereby incorporated by reference) describes a method for increasing the rate of healing of wound tissue, comprising the application to such tissue of angiotensin II (Ang II) in an

amount which is sufficient for said increase. The application of AII to wound tissue significantly increases the rate of wound healing, leading to a more rapid re-epithelialization and tissue repair. The term AII refers to an octapeptide present in humans and other species having the sequence Asp-Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:1]. The biological formation of angiotensin is initiated by the action of renin on the plasma substrate angiotensinogen (Clouston et al., *Genomics* 2:240-248 (1988); Kageyama et al., *Biochemistry* 23:3603-3609; Ohkubo et al., *Proc. Natl. Acad. Sci.* 80:2196-2200 (1983); each reference hereby incorporated in its entirety). The substance so formed is a decapeptide called angiotensin I (AI) which is converted to AII by the angiotensin converting enzyme (ACE) which removes the C-terminal His-Leu residues from AI [SEQ ID NO: 37]. AII is a known pressor agent and is commercially available.

Studies have shown that AII increases mitogenesis and chemotaxis in cultured cells that are involved in wound repair, and also increases their release of growth factors and extracellular matrices (diZerega, U.S. Patent No. 5,015,629; Dzau et. al., *J. Mol. Cell. Cardiol.* 21:S7 (Supp III) 1989; Berk et. al., *Hypertension* 13:305-14 (1989); Kawahara, et al., *BBRC* 150:52-9 (1988); Nafilan, et al., *J. Clin. Invest.* 83:1419-23 (1989); Taubman et al., *J. Biol. Chem.* 264:526-530 (1989); Nakahara, et al., *BBRC* 184:811-8 (1992); Stouffer and Owens, *Circ. Res.* 70:820 (1992); Wolf, et al., *Am. J. Pathol.* 140:95-107 (1992); Bell and Madri, *Am. J. Pathol.* 137:7-12 (1990). In addition, AII was shown to be angiogenic in rabbit corneal eye and chick chorioallantoic membrane models (Fernandez, et al., *J. Lab. Clin. Med.* 105:141 (1985); LeNoble, et al., *Eur. J. Pharmacol.* 195:305-6 (1991). Additionally, AII and angiotensin III analogs and fragments, and AII Type 2 receptor agonists thereof have been shown to be effective in wound healing. (U.S. Patent Nos. 5,629,292; 5,716,935; International Application Nos.

WO 94/10502; WO 94/10503; WO 95/14764; WO 95/08565; WO 95/08337; WO 96/09747; WO 96/39164; WO 97/23461; WO 98/26437; all references hereby incorporated in their entirety.)

Angiotensin II is also known as a potent stimulator of angiogenesis (Fernandez et al., *J. Lab. Clin. Med.* 105:141-145 (1985)), and has been shown to activate collateral
5 circulation via preformed blood vessels in rat kidneys (Fernandez et al., *Am. J. Physiol.* 243:H869-H875 (1982)).

The effect of AII on a given cell type has been hypothesized to be dependent, in part, upon the AII receptor subtypes the cell expresses (Shanugam et al., *Am. J. Physiol.*
10 268:F922-F930 (1995); Helin et al., *Annals of Medicine* 29:23-29 (1997); Bedecs et al., *Biochem J.* 325:449-454 (1997)). These studies have shown that AII receptor subtype expression is a dynamic process that changes during development, at least in some cell types (*Id.*). AII activity is typically modulated by either or both the AT1 and AT2 AII receptors. However, AII has recently been shown to stimulate proliferation of primary
15 human keratinocytes via a non-AT1, non-AT2 receptor. (Steckelings et al., *Biochem. Biophys. Res. Commun.* 229:329-333 (1996)). These results underscore the cell-type (ie: based on receptor expression) specific nature of AII activity.

Other data suggests that the AII fragment AII(1-7) acts through a receptor(s) that is distinct from the AT1 and AT2 receptors which modulate AII activity. (Ferrario et al.,
20 *J. Am. Soc. Nephrol.* 9:1716-1722 (1998); Iyer et al., *Hypertension* 31:699-705 (1998); Freeman et al., *Hypertension* 28:104 (1996); Ambuhl et al., *Brain Res. Bull.* 35:289 (1994). Thus, AII(1-7) activity, on a particular cell type cannot be predicted based solely on the effect of AII on the same cell type.

However, based on all of the above, it is unknown whether the use of angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, AII analogues, AII fragments or analogues thereof or AII AT₂ type 2 receptor (AT₂) agonists would be effective in stimulating blood flow to ischemic tissue. The
5 identification of AII analogues and fragments that stimulate blood flow with fewer side effects than AII would be extremely beneficial.

A peptide agonist selective for the AT₂ receptor (AII has 100 times higher affinity for AT₂ than AT₁) has been identified. This peptide is p-aminophenylalanine 6-AII ["(p-NH₂-Phe)6-AII"], Asp-Arg-Val-Tyr-Ile-Xaa-Pro-Phe [SEQ ID NO.36] wherein
10 Xaa is p-NH₂-Phe (Speth and Kim, BBRC 169:997-1006 (1990). This peptide gave binding characteristics comparable to AT₂ antagonists in the experimental models tested (Catalioto, et al., *Eur. J. Pharmacol.* 256:93-97 (1994); Bryson, et al., *Eur. J. Pharmacol.* 225:119-127 (1992).

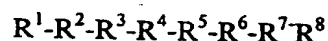
The effects of AII receptor and AII receptor antagonists have been examined in
15 two experimental models of vascular injury and repair which suggest that both AII receptor subtypes (AT₁ and AT₂) play a role in wound healing (Janiak et al., *Hypertension* 20:737-45 (1992); Prescott, et al., *Am. J. Pathol.* 139:1291-1296 (1991); Kauffman, et al., *Life Sci.* 49:223-228 (1991); Viswanathan, et al., *Peptides* 13:783-786 (1992); Kimura, et al., *BBRC* 187:1083-1090 (1992).

20 Many studies have focused upon AII(1-7) (AII residues 1-7) or other fragments of AII to evaluate their activity. AII(1-7) elicits some, but not the full range of effects elicited by AII. Pfeilschifter, et al., *Eur. J. Pharmacol.* 225:57-62 (1992); Jaiswal, et al., *Hypertension* 19(Supp. II):II-49-II-55 (1992); Edwards and Stack, *J. Pharmacol. Exper. Ther.* 266:506-510 (1993); Jaiswal, et al., *J. Pharmacol. Exper. Ther.* 265:664-673

(1991); Jaiswal, et al., *Hypertension* 17:1115-1120 (1991); Portsi, et a., *Br. J. Pharmacol.* 111:652-654 (1994).

As hereinafter defined, a preferred class of AT₂ agonists for use in accordance with the present invention comprises angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, AII analogues, AII fragments or analogues thereof or AII AT₂ type 2 receptor agonists having p-NH-Phe in a position corresponding to a position 6 of AII. In addition to peptide agents, various nonpeptidic agents (e.g., peptidomimetics) having the requisite AT₂ agonist activity are further contemplated for use in accordance with the present invention.

The active AII analogues, fragments of AII and analogues thereof of particular interest in accordance with the present invention comprise a sequence consisting of at least three contiguous amino acids of groups R¹-R⁸ in the sequence of general formula I



in which R¹ and R² together form a group of formula



wherein X is H or a one to three peptide group, or is absent,

R^A is suitably selected from H, Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, Me²Gly, Pro, Bet, Glu(NH₂), Gly, Asp(NH₂) and Suc,

R^B is suitably selected from Arg, Lys, Ala, Orn, Citron, Ser(Ac), Sar, D-Arg and D-Lys;

R³ is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

R⁴ is selected from the group consisting of Tyr, Tyr(PO₃)₂, Thr, Ala, Ser, homoSer and azaTyr;

R⁵ is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

5 R⁶ is His, Arg or 6-NH₂-Phe;

R⁷ is Pro or Ala; and

R⁸ is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R⁴ as a terminal Tyr group, or is absent,

wherein the active agent is not AII.

10 Compounds falling within the category of AT2 agonists useful in the practice of the invention include the AII analogues set forth above subject to the restriction that R⁶ is p-NH₂-Phe.

Particularly preferred combinations for R^A and R^B are Asp-Arg, Asp-Lys, Glu-Arg and Glu-Lys. Particularly preferred embodiments of this class include the
15 following: AIII or AII(2-8), Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:2]; AII(3-8), also known as des1-AIII or AIV, Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:3]; AII(1-7), Asp-Arg-Val-Tyr-Ile-His-Pro [SEQ ID NO:4]; AII(2-7), Arg-Val-Tyr-Ile-His-Pro [SEQ ID NO:5]; AII(3-7), Val-Tyr-Ile-His-Pro [SEQ ID NO:6]; AII(5-8), Ile-His-Pro-Phe [SEQ ID NO:7]; AII(1-6), Asp-Arg-Val-Tyr-Ile-His [SEQ ID NO:8]; AII(1-5), Asp-Arg-Val-Tyr-Ile [SEQ ID NO:9]; AII(1-4), Asp-Arg-Val-Tyr [SEQ ID NO:10]; and AII(1-3), Asp-Arg-Val [SEQ ID NO:11]. Other preferred embodiments include: Arg-norLeu-Tyr-Ile-His-Pro-Phe [SEQ ID NO:12] and Arg-Val-Tyr-norLeu-His-Pro-Phe [SEQ ID NO:13]. Still another preferred embodiment encompassed within the scope of the invention is a peptide having the sequence Asp-Arg-Pro-Tyr-Ile-His-Pro-Phe [SEQ ID
20

NO:31]. AII(6-8), His-Pro-Phe [SEQ ID NO:14] and AII(4-8), Tyr-Ile-His-Pro-Phe [SEQ ID NO:15] were also tested and found not to be effective.

In a particularly preferred embodiment, the active compounds of the present invention are selected from those comprising the following general formula:

5 R1-Arg-R2-R3-R4-His-Pro-R5, wherein

R1 is Asp or is absent;

R2 is selected from the group consisting of Val, Ala, Ile, Pro, Lys, Norleu, and Leu;

R3 is selected from the group consisting of Ala, Tyr, and Tyr(PO₃)₂; and

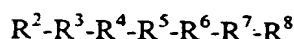
10 R4 is selected from the group consisting of Val, Ala, Ile, Norleu, and Leu;

R5 is Phe, Ile, or is absent;

wherein the compound is not AII.

In a most particularly preferred embodiment, the active compound is selected from the group consisting of SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, and SEQ ID NO:42.

Another class of compounds of particular interest in accordance with the present invention are those of the general formula II



20 in which R² is selected from the group consisting of H, Arg, Lys, Ala, Orn, Citron, Ser(Ac), Sar, D-Arg and D-Lys;

R³ is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

R^4 is selected from the group consisting of Tyr, Tyr(PO₃)₂, Thr, Ser, homoSer, Ala, and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

5 R^6 is His, Arg or 6-NH₂-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr.

A particularly preferred subclass of the compounds of general formula II has the formula

10 R^2 - R^3 -Tyr- R^5 -His-Pro-Phe [SEQ ID NO:16]

wherein R^2 , R^3 and R^5 are as previously defined. Particularly preferred is angiotensin III of the formula Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:2]. Other preferred compounds include peptides having the structures Arg-Val-Tyr-Gly-His-Pro-Phe [SEQ ID NO:17] and Arg-Val-Tyr-Ala-His-Pro-Phe [SEQ ID NO:18]. The
15 fragment AII(4-8) was ineffective in repeated tests; this is believed to be due to the exposed tyrosine on the N-terminus.

In the above formulas, the standard three-letter abbreviations for amino acid residues are employed. In the absence of an indication to the contrary, the L-form of the amino acid is intended. Other residues are abbreviated as follows:

20 TABLE 1

Abbreviation for Amino Acids

Me ² Gly	N,N-dimethylglycyl
---------------------	--------------------

Bet	1-carboxy-N,N,N-trimethylmethanaminium hydroxide inner salt (betaine)
Suc	Succinyl
Phe(Br)	p-bromo-L-phenylalanyl
azaTyr	aza- α' -homo-L-tyrosyl
Acpc	1-aminocyclopentane carboxylic acid
Aib	2-aminoisobutyric acid
Sar	N-methylglycyl (sarcosine)
Om	Ornithine

It has been suggested that AII and its analogues adopt either a *gamma* or a *beta* turn (Regoli, et al., *Pharmacological Reviews* 26:69 (1974)). In general, it is believed that neutral side chains in position R³, R⁵ and R⁷ may be involved in maintaining the appropriate distance between active groups in positions R⁴, R⁶ and R⁸ primarily responsible for binding to receptors and/or intrinsic activity. Hydrophobic side chains in positions R³, R⁵ and R⁸ may also play an important role in the whole conformation of the peptide and/or contribute to the formation of a hypothetical hydrophobic pocket.

Appropriate side chains on the amino acid in position R² may contribute to affinity of the compounds for target receptors and/or play an important role in the conformation of the peptide. For this reason, Arg and Lys are particularly preferred as R².

For purposes of the present invention, it is believed that R³ may be involved in the formation of linear or nonlinear hydrogen bonds with R⁵ (in the *gamma* turn model) or R⁶ (in the *beta* turn model). R³ would also participate in the first turn in a *beta* antiparallel structure (which has also been proposed as a possible structure). In contrast

to other positions in general formula I, it appears that beta and gamma branching are equally effective in this position. Moreover, a single hydrogen bond may be sufficient to maintain a relatively stable conformation. Accordingly, R³ may suitably be selected from Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr. R³ may also suitably be
5 Lys.

With respect to R⁴, conformational analyses have suggested that the side chain in this position (as well as in R³ and R⁵) contribute to a hydrophobic cluster believed to be essential for occupation and stimulation of receptors. Thus, R⁴ is preferably selected from Tyr, Thr, Tyr (PO₃)₂, homoSer, Ser and azaTyr. In this position, Tyr is particularly
10 preferred as it may form a hydrogen bond with the receptor site capable of accepting a hydrogen from the phenolic hydroxyl (Regoli, et al. (1974), *supra*). R⁴ may also suitably be Ala.

In position R⁵, an amino acid with a β aliphatic or alicyclic chain is particularly desirable. Therefore, while Gly is suitable in position R⁵, it is preferred that the amino
15 acid in this position be selected from Ile, Ala, Leu, norLeu, Gly and Val.

In the angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII analogues, fragments and analogues of fragments of particular interest in accordance with the present invention, R⁶ is His, Arg or 6-NH₂-Phe. The unique properties of the imidazole ring of histidine (e.g., ionization at physiological pH, ability to act as proton
20 donor or acceptor, aromatic character) are believed to contribute to its particular utility as R⁶. For example, conformational models suggest that His may participate in hydrogen bond formation (in the *beta* model) or in the second turn of the antiparallel structure by influencing the orientation of R⁷. Similarly, it is presently considered that R⁷ should be Pro in order to provide the most desirable orientation of R⁸. In position R⁸, both a

hydrophobic ring and an anionic carboxyl terminal appear to be particularly useful in binding of the analogues of interest to receptors; therefore, Tyr and especially Phe are preferred for purposes of the present invention. R⁸ may also suitably be Ile.

Analogues of particular interest include the following:

5 TABLE 2

Angiotensin II Analogues

AII Analogue Name	Amino Acid Sequence	Sequence Identifier
Analogue 1	Asp-Arg-Val-Tyr-Val-His-Pro-Phe	SEQ ID NO: 19
Analogue 2	Asn-Arg-Val-Tyr-Val-His-Pro-Phe	SEQ ID NO: 20
Analogue 3	Ala-Pro-Gly-Asp-Arg-Ile-Tyr-Val-His-Pro-Phe	SEQ ID NO: 21
Analogue 4	Glu-Arg-Val-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 22
Analogue 5	Asp-Lys-Val-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 23
Analogue 6	Asp-Arg-Ala-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 24
Analogue 7	Asp-Arg-Val-Thr-Ile-His-Pro-Phe	SEQ ID NO: 25
Analogue 8	Asp-Arg-Val-Tyr-Leu-His-Pro-Phe	SEQ ID NO: 26
Analogue 9	Asp-Arg-Val-Tyr-Ile-Arg-Pro-Phe	SEQ ID NO: 27
Analogue 10	Asp-Arg-Val-Tyr-Ile-His-Ala-Phe	SEQ ID NO: 28
Analogue 11	Asp-Arg-Val-Tyr-Ile-His-Pro-Tyr	SEQ ID NO: 29
Analogue 12	Pro-Arg-Val-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 30
Analogue 13	Asp-Arg-Pro-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 31
Analogue 14	Asp-Arg-Val-Tyr(PO ₃) ₂ -Ile-His-Pro-Phe	SEQ ID NO: 32
Analogue 15	Asp-Arg-norLeu-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 33
Analogue 16	Asp-Arg-Val-Tyr-norLeu-His-Pro-Phe	SEQ ID NO: 34
Analogue 17	Asp-Arg-Val-homoSer-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 35

The polypeptides of the instant invention may be synthesized by any conventional method, including, but not limited to, those set forth in J. M. Stewart and J.

- 10 D. Young, *Solid Phase Peptide Synthesis*, 2nd ed., Pierce Chemical Co., Rockford, Ill. (1984) and J. Meienhofer, *Hormonal Proteins and Peptides*, Vol. 2, Academic Press, New York, (1973) for solid phase synthesis and E. Schroder and K. Lubke, *The Peptides*, Vol. 1, Academic Press, New York, (1965) for solution synthesis. The disclosures of the foregoing treatises are incorporated by reference herein.

In general, these methods involve the sequential addition of protected amino acids to a growing peptide chain (U.S. Patent No. 5,693,616, herein incorporated by reference in its entirety). Normally, either the amino or carboxyl group of the first amino acid and any reactive side chain group are protected. This protected amino acid is then
5 either attached to an inert solid support, or utilized in solution, and the next amino acid in the sequence, also suitably protected, is added under conditions amenable to formation of the amide linkage. After all the desired amino acids have been linked in the proper sequence, protecting groups and any solid support are removed to afford the crude polypeptide. The polypeptide is desalted and purified, preferably chromatographically, to
10 yield the final product.

Preferably, peptides are synthesized according to standard solid-phase methodologies, such as may be performed on an Applied Biosystems Model 430A peptide synthesizer (Applied Biosystems, Foster City, Calif.), according to manufacturer's instructions. Other methods of synthesizing peptides or peptidomimetics,
15 either by solid phase methodologies or in liquid phase, are well known to those skilled in the art.

In one aspect, the present invention provides methods and kits to increase blood flow to ischemic tissue, comprising the administration of angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, angiotensin II (AII) analogues,
20 AII fragments or analogues thereof or AII AT₂ type 2 receptor agonists (hereinafter referred to as the "active agents").

According to the invention, an area of ischemic tissue is treated in vivo to maintain viability of that area for a sustained period of time to salvage the area. An effective dose of the active agents is applied to the ischemic tissue, preferably after

ischemia, although it can also be applied when there is an indication of impending ischemia.

The ischemic tissue is suitably any tissue that can benefit by an increased blood flow to the tissue to reverse or prevent the adverse effects of ischemia on the tissue. In preferred embodiments, the tissue is selected from skin and heart. In a further preferred embodiment, the active agents can be used to stimulate blood flow to tissue grafts, whether artificial or transplanted.

The active agents may be administered by any suitable route, including orally, parentally, by inhalation spray, rectally, or topically in dosage unit formulations containing conventional pharmaceutically acceptable carriers, adjuvants, and vehicles. The term parenteral as used herein includes, subcutaneous, intravenous, intraarterial, intramuscular, intrasternal, intracardiac, intratendinous, intraspinal, intracranial, intrathoracic, infusion techniques or intraperitoneally. Furthermore, angiotensinogen can be administered by gene therapy techniques.

The active agents of the invention may be made up in a solid form (including granules, powders or suppositories) or in a liquid form (*e.g.*, solutions, suspensions, or emulsions). The compounds of the invention may be applied in a variety of solutions. Suitable solutions for use in accordance with the invention are sterile, dissolve sufficient amounts of the peptide, and are not harmful for the proposed application. In this regard, the compounds of the present invention are very stable but are hydrolyzed by strong acids and bases. The compounds of the present invention are soluble in organic solvents and in aqueous solutions at pH 5-8.

The active agents may be subjected to conventional pharmaceutical operations such as sterilization and/or may contain conventional adjuvants, such as preservatives, stabilizers, wetting agents, emulsifiers, buffers etc.

The active agents of the invention can be used alone or in a combination of active agents, or may be used in combination with other agents that increase blood flow to ischemic tissue, either via production of new blood vessels (including, but not limited to AII, acidic and basic fibroblast growth factor, transforming growth factor alpha and beta, tumor necrosis factor, platelet-derived growth factor, vascular endothelial growth factor, angiogenin, and haptoglobin), or by stimulating blood flow to ischemic tissue through pre-existing blood vessels, including but not limited to fibroblast growth factor.

For administration, the active agents are ordinarily combined with one or more adjuvants appropriate for the indicated route of administration. The compounds may be admixed with lactose, sucrose, starch powder, cellulose esters of alkanolic acids, stearic acid, talc, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulphuric acids, acacia, gelatin, sodium alginate, polyvinylpyrrolidone, and/or polyvinyl alcohol, and tableted or encapsulated for conventional administration. Alternatively, the compounds of this invention may be dissolved in saline, water, polyethylene glycol, propylene glycol, carboxymethyl cellulose colloidal solutions, ethanol, corn oil, peanut oil, cottonseed oil, sesame oil, tragacanth gum, and/or various buffers. Other adjuvants and modes of administration are well known in the pharmaceutical art. The carrier or diluent may include time delay material, such as glyceryl monostearate or glyceryl distearate alone or with a wax, or other materials well known in the art.

Formulations suitable for topical administration include liquid or semi-liquid preparations suitable for penetration through the skin (e.g., liniments, lotions, ointments, creams, or pastes) and drops suitable for administration to the eye, ear, or nose.

The dosage regimen for increasing blood flow to ischemic tissue with the active agents of the invention is based on a variety of factors, including the type of injury, the age, weight, sex, medical condition of the individual, the severity of the condition, the route of administration, and the particular compound employed. Thus, the dosage regimen may vary widely, but can be determined routinely by a physician using standard methods. Dosage levels of the order of between 0.1 ng/kg and 10 mg/kg body weight active agent per body weight are useful for all methods of use disclosed herein.

The treatment regime will also vary depending on the condition of the subject, based on a variety of factors, including the type of injury, the age, weight, sex, medical condition of the individual, the severity of the condition, the route of administration, and the particular compound employed. For example, an active agents is administered to a patient as soon as possible after myocardial infarction for up to 30 days. The therapy is administered for 1 to 6 times per day at dosages as described above.

In a preferred embodiment, the active agent is administered subcutaneously. A suitable subcutaneous dose of active ingredient of active agent is preferably between about 0.1 ng/kg and about 10 mg/kg administered twice daily for a time sufficient to increase blood flow to ischemic tissue. In a more preferred embodiment, the concentration of active agent is between about 100 ng/kg body weight and about 10.0 mg/kg body weight. In a most preferred embodiment, the concentration of active agent is between about 10 μ g/kg body weight and about 10.0 mg/kg body weight. This dosage regimen maximizes the therapeutic benefits of the subject invention while minimizing

the amount of antagonist needed. Such an application minimizes costs as well as possible deleterious side effects.

For subcutaneous administration, the active ingredient may comprise from 0.0001% to 10% w/w, *e.g.*, from 1% to 2% by weight of the formulation, although it may
5 comprise as much as 10% w/w, but preferably not more than 5% w/w, and more preferably from 0.1% to 1% of the formulation.

In a preferred embodiment, a catheter is placed into the coronary artery of a subject between about immediately after to about 24 hours after myocardial infarction and injecting an effective amount of the active agent into the heart of the subject. The
10 concentration of active agent injected is between about 100 ng/kg body weight and about 10.0 mg/kg body weight, as described above. The injection can be repeated as needed to stimulate increased blood flow in the existing collateral circulation. Injections can also be by other routes, including but not limited to by catheter such via arterial angiography, intracoronary injection, or in a cardioplegic solution by the aortic route. Increased
15 collateral blood flow is determined by standard procedures. For example, collateral flow can be quantified during Chromonar-induced maximum vasodilation and expressed as an ischemic/normal zone (IZ/NZ) ratio. (U.S. Patent No. 5,244,460 incorporated by reference herein in its entirety) Alternatively, collateral blood flow can be quantitated using radioactive microspheres using a reference flow technique (Dole, et al, *Am. J.*
20 *Physiol.*, 1982; 243:H371-H378).

In another preferred embodiment of the present invention, the active agent is administered topically. Suitable topical doses and active ingredient concentration in the formulation are as described for subcutaneous administration.

In a further preferred embodiment of all of the aspects of the invention, the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, and SEQ ID NO:42.

In a further aspect, the present invention provides kits to increase blood flow to ischemic tissue, wherein the kits comprise an effective amount of active agent for increasing blood flow to ischemic tissue, and instructions for using the amount effective of active agent as a therapeutic. In a preferred embodiment, the kit further comprises a pharmaceutically acceptable carrier, such as those adjuvants described above. In another preferred embodiment, the kit further comprises a means for delivery of the active agent to a patient. Such devices include, but are not limited to syringes, matrical or micellar solutions, bandages, wound dressings, aerosol sprays, lipid foams, transdermal patches, topical administrative agents, polyethylene glycol polymers, carboxymethyl cellulose preparations, crystalloid preparations (e.g., saline, Ringer's lactate solution, phosphate-buffered saline, etc.), viscoelastics, polyethylene glycols, and polypropylene glycols. The means for delivery may either contain the effective amount of active agent, or may be separate from the compounds, which are then applied to the means for delivery at the time of use.

The present invention may be better understood with reference to the accompanying examples that are intended for purposes of illustration only and should not be construed to limit the scope of the invention.

5 *Example 1: Engraftment Of Living-Skin Equivalent*

Male Swiss nude mice (22-24 g) were anesthetized with an intramuscular injection of Ketaset/Rompun and a 1 cm x 1 cm full thickness skin excision was made on their dorsal surface. Living skin equivalent (LSE) was placed in the defect and trimmed
10 with microscissors so that no gap was observed between the edges of the mouse skin and the LSE. The mice were divided into 6 groups based upon the peptide tested: (0 [LRS-Dextrose], or 1.0 mg/ml AII or AII analogues, AII (1-7) [SEQ ID NO:4]; Ala4AII [SEQ ID NO:18]; Pro3AII [SEQ ID NO:31]; and Ile8AII [SEQ ID NO: 42]; see Table 1) and the LSE was soaked for 15 minutes in lactated Ringers solution with 5% dextrose with or
15 without peptides prior to placement. After the graft was placed, the dorsal surface of the mouse was covered by petrolatum-embedded gauze followed by two adhesive bandages. After recovery from anesthesia, the mice were returned to their individual cages and observed daily until euthanasia. The mice received intramuscular analgesia for the first three days after surgery. No mouse lost its bandages prior to necropsy or bandage
20 removal on day 7. The mice were necropsied on day 7. At necropsy, the degree of graft take and the appearance of the grafted tissue was noted prior to placement of the biopsy in 10% buffered formalin in preparation for paraffin embedding and section for hematoxylin and eosin staining. The number of endothelial cells and vascular channels were assessed microscopically and the data are presented in Figures 1 and 2. These data
25 demonstrate that AII and the AII analogue-treated LSE increased both angiogenesis

(vascular channel formation) and endothelial cell production in graft tissue compared to untreated LSE.

Table 1. Designation for Analogues/Fragments used in Examples 1-3

5	1GD	Ala ⁴ -AII(1-7)	DRVAIHP	SEQ ID NO:38
	2GD	Pro ³ -AII(1-7)	DRPYIHP	SEQ ID NO:39
	5GD	Lys3-AII(1-7)	DRKYIHP	SEQ ID NO:40
	9GD:	NorLeu3-AII(1-7)	DR(nor)YIHP	SEQ ID NO:41
10	AII(1-7)		DRVYIHP	SEQ ID NO:4
	AII		DRVYIHPF	SEQ ID NO. 1
		Ala4-AIII	RVAHPF	SEQ ID NO:18
		Pro3-AII	DRVYIHPF	SEQ ID NO:31
15		Ile8-AII	DRVYIHPI	SEQ ID NO:42

Example 2: Partial Thickness Thermal Injury Model

Animals

Thirty-six male Hartley guinea pigs weighing approximately 500 grams were purchased from Charles Rivers Laboratory (Charles Rivers Mass.). The guinea pigs were housed in a 12:12 hour light: dark, cycle in the USC Vivaria. Food and water were available *ad libitum*.

Surgical Procedures

The guinea pigs were anesthetized by intramuscular injection of 14 mg/kg Rompun and 130 mg/kg Ketamine. Hair was then removed from the dorsal surface through shaving with animal clippers followed by treatment with a thioglycollate depilatory. After hair removal the area was scrubbed with betadine twice followed by 70% ethanol. Two burns were produced on each guinea pig with an 18 mm solid brass rod, which was warmed in a 75°C water bath. One end of the brass rod was placed on

the back of the guinea pig for 50 seconds. This procedure was repeated with different brass rods for each animal and each burn.

Each burn was treated with 10% low-viscosity CMC with and without 1 mg/ml AII, AII analogue or fragment (0 [LRS-Dextrose], or 1.0 mg/ml AII or AII analogues, AII (1-7) [SEQ ID NO:4]; Ala4AII [SEQ ID NO:18]; Pro3AII [SEQ ID NO:31]; or Ile8AII [SEQ ID NO: 42]) in 0.05 mol/L phosphate buffer, pH 7.3, and was individually dressed with a Hilltop Chamber and covered with Tegaderm. The bandages were checked and changed daily for the first 5 days and every other day until necropsy on day 7 after injury. The guinea pigs were given 20 µg/kg bupronex (buprenorphine hydrochloride) intramuscularly for pain on the day of injury and the first 3 days after injury.

Validation with India Ink Injection

Initial studies were conducted in which the depth of the burn was evaluated with intraaortic injection of India Ink. One or 2 days after initiation of the thermal injury, the guinea pigs were anesthetized with intramuscular ketamine and rompun. The ascending thoracic aorta was cannulated, and 60 ml India Ink was injected through the cannula under 500 mm Hg. This pressure was used to ensure filling of all patent vessels. After the India Ink was injected the animals were sacrificed, and the injury sites were excised and placed in 10% buffered formalin and prepared for histologic evaluation. The histologic sections from these preparations were examined to evaluate the depth of necrosis and capillary ischemia.

Histologic Evaluation

On day 7 after thermal injury, the guinea pigs were euthanized, the burned areas were excised en bloc and the tissues were placed in 10% buffered formaldehyde solution

overnight. The tissues were embedded in paraffin, and 5 μ m sections were prepared. The sections were processed for immunohistochemical analysis with a primary antibody to cyclin (MIB-1) (AMAC, Westbrook, MA) followed by recognition of the primary antibody with a DAKO kit (DAKO, Santa Barbara, CA) used as described below.

5 The paraffin-embedded sections were baked in an oven overnight at 60° C. Deparaffinization was performed by four 5-minute incubations in "fresh" xylene followed by two 5-minute incubations with 100% ethanol, two 5-minute incubations with 95% ethanol, and one 5-minute incubation with H₂O. Endogenous peroxidase activity was then quenched with 0.3% H₂O₂ in H₂O. Antigen retrieval was performed
10 following the protocol of Shi et al. with minor modifications. In brief, the slides were placed in plastic coplin jars in 5% urea in 0.1 mol/L Tris buffer (pH 9.0). The slides were then placed in a microwave oven and set at maximal power for 3 minutes. The antigen retrieval solution was replenished with distilled water when evaporation was excessive and were microwaved again for 5 minutes at 50% power. The slides were then
15 allowed to cool for 10 minutes and were placed in phosphate-buffered saline solution (PBS) for 5 minutes.

Immunohistochemical staining was performed with the avidin-biotin-peroxidase conjugate method with some modifications. The slides were placed in a PBS bath for 5 minutes and then laid in humidified incubation chambers. Blocking for nonspecific
20 antibody binding was performed by incubating the sections in 5% horse serum in PBS. The solution was decanted and replaced with the solution of primary antibody. The primary antibody, MIB-1, was used at a dilution of 1:100 in PBS and was incubated for 60 minutes at room temperature. The slides were washed with PBS for 5 minutes and were then incubated with the secondary biotinylated horse-anti-mouse antibody. After a

5-minute wash in PBS the avidin-biotin complex was applied to slides at a dilution of 1:100 in PBS at room temperature and was incubated for 1 hour.

After a final rinse in PBS, the sections were incubated in 0.06% 3,3'-diaminobenzidine in PBS with 0.03% hydrogen peroxide for 5 minutes. After a counterstain was performed in modified Harris' hematoxylin-eosin the sections were
5 dehydrated and coverslipped with Permount.

With an Olympus Vanox-S AH-2 dissecting microscope and a magnification power of 100x, each section of the biopsy specimen was separated into either areas on the burn edge or the actual burn areas. The area of the burn was excised and serially cut
10 into three to five sections 4 to 5 mm in thickness. The entire area of the burn and edge of the burn was embedded and examined histologically. In each section four to six consecutive medium-power fields (mpf, 100x) were evaluated. The cells that stained with the MIB-1 antibody were a distinct brown color. All stained cells located within the hair follicles of the biopsy sections were counted. To count the MIB-1 stained cells each
15 section on the slide was separated into individual medium power fields (mpf). Each field was then determined to be either a section on the edge of the burn or a part of the burn area itself. An edge was indicated by a positive stain showing brown epithelial cells along the edge of the section. A burn area was indicated by an absence of brown staining cells along the edge. The brown cells located within each hair follicle were
20 counted one at a time under mpf magnification. To move to the next mpf a landmark was established, and the slide was then moved to the next adjacent field.

All animals survived the burn procedures and did not demonstrate evidence of significant discomfort throughout the study interval. The depth of the burns was determined to be deep partial-thickness to full-thickness by (1) analysis of vessel patency

with intraaortic injection of India Ink and (2) the appearance of the cells in the hair follicles by microscopic analysis of hematoxylin-eosin-stained sections. This analysis revealed most of the preexisting blood vessels and cells were destroyed in the burn site, although the injuries did not extend through the panniculus carnosus.

5 Figure 3 demonstrates the effect of AII and the same AII analogues used in Example 1 on angiogenesis after thermal injury. These data demonstrate that treatment of the burn areas with the AII and AII analogues produces an increased blood flow response compared to control, with the AII analogues showing an increased blood flow response compared to AII.

10 *Example 3. Effect of Angiotensin Peptides on the Formation of Collateral Circulation in Ischemic Myocardial Tissue*

15 Rabbits underwent a surgical procedure under intramuscular anesthesia (Ketamine and Rompum) after shaving with animal clippers and preparation with betadine and isopropyl alcohol. A midline sternotomy was performed. After exposure of the pericardial sac, a three centimeter incision was made into the pericardium. After visualization of the epicardial surface, two coronary arteries, the left circumflex and the
20 left anterior descending arteries, were exposed and ligated by 4-0 Vicryl suture. Vehicle (10% Hydron, 60% ethanol and 1% polyethylene glycol), with or without peptide (1 mg/ml, 0.05 ml) was injected in the cardiac muscle distal to the site of coronary occlusion. The sternum was then closed with 2-0 Silk. The muscle and skin were then closed with 3-0 Dexon II suture. Starting at 7 days after surgery, the animals were
25 euthanized and necropsies performed. The number of blood vessels present in the infarct site was assessed grossly and by microscopic evaluation. The presence of a blood

vessel was defined as a channel lined with endothelial cells that contained red blood cells (indicating that the vessels had a blood source).

All peptides (AII, AII(1-7), 1GD, 2GD, 5GD and 9GD: see table 2) tested increase vascularization of the infarct site. The increases ranged from approximately 4 fold to approximately 14 fold increase in the formation of collateral circulation after ligation of the artery. The data collected from microscopic examination of multiple tissue sections are in Table 2.

Table 2. Effect of Angiotensin Peptides on the Formation of Collateral Circulation in Ischemic Myocardial Tissue

Treatment	# of Animals	Mean \pm SEM	P Value
Hydron	7	5.67 \pm 0.74	
1GD	4	23.4 \pm 8.6	0.020
2GD	2	31.2 \pm 5.62	<0.001
5GD	2	24.55 \pm 2.34	<0.001
9GD	3	21.2 \pm 2.8	<0.001
AII	4	29.8 \pm 12.3	<0.001
AII(1-7)	2	80.6 \pm 4.5	<0.001

10

The methods and kits of the present invention, by increasing blood flow to ischemic tissue, significantly enhance the utility of presently available treatments for ischemia, and particularly benefit treatments for skin and heart ischemia, and significantly benefit tissue grafting by increasing blood flow to tissue grafts.

It is to be understood that the invention is not to be limited to the exact details of operation, or to the exact compounds, compositions, methods, procedures or embodiments shown and described, as obvious modifications and equivalents will be apparent to one skilled in the art, and the invention is therefore to be limited only by the full scope of the appended claims.

20

We claim:

1. A method for increasing blood flow to ischemic tissue comprising the administration of an amount effective for increasing blood flow to ischemic tissue of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups R^1 - R^8 in the sequence of general formula I



in which R^1 and R^2 together form a group of formula



wherein X is H or a one to three peptide group, or is absent,

10 R^A is suitably selected from H, Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, Me²Gly, Pro, Bet, Glu(NH₂), Gly, Asp(NH₂) and Suc;

R^B is suitably selected from Arg, Lys, Ala, Orn, Citron, Ser(Ac), Sar, D-Arg and D-Lys;

15 R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

R^4 is selected from the group consisting of Tyr, Tyr(PO₃)₂, Thr, Ala, Ser, homoSer and azaTyr;

20 R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH₂-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group, or is absent;

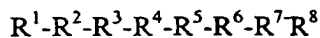
and wherein the compound is not AII.

2. The method of claim 1 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, 5 SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, and SEQ ID NO:42.

3. The method of claim 1 wherein the ischemic tissue is selected from skin, heart, and tissue grafts.

4. The method of claim 1, further comprising administration of an amount effective for increasing blood flow to ischemic tissue of at least one compound selected from the 15 group consisting of AII, acidic fibroblast growth factor, basic fibroblast growth factor, transforming growth factor alpha, transforming growth factor beta, tumor necrosis factor, platelet-derived growth factor, vascular endothelial growth factor, angiogenin, and haptoglobin.

5. A kit for increasing blood flow to ischemic tissue, comprising:
20 (a) an amount effective for increasing blood flow to ischemic tissue of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups R^1 - R^8 in the sequence of general formula I



in which R^1 and R^2 together form a group of formula



wherein X is H or a one to three peptide group, or is absent,

R^A is suitably selected from H, Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, Me²Gly, Pro, Bet, Glu(NH₂), Gly, Asp(NH₂) and Suc;

R^B is suitably selected from Arg, Lys, Ala, Orn, Citron, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

R^4 is selected from the group consisting of Tyr, Tyr(PO₃)₂, Thr, Ala, Ser, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH₂-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group, or is absent; wherein the compound is not AII; and

(b) instructions for using the amount effective of active agent for increasing blood flow to ischemic tissue.

6. The kit of claim 5 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ

ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, 5 SEQ ID NO:40, SEQ ID NO:41, and SEQ ID NO:42.

7. The kit of claim 5 further comprising a means for delivery of the active agent.

8. The kit of claim 5 further comprising an amount effective for increasing blood flow to ischemic tissue of at least one compound selected from the group consisting of AII, acidic fibroblast growth factor, basic fibroblast growth factor, transforming growth 10 factor alpha, transforming growth factor beta, tumor necrosis factor, platelet-derived growth factor, vascular endothelial growth factor, angiogenin, and haptoglobin.

9. A method for increasing blood flow to ischemic tissue comprising the administration of an amount effective for increasing blood flow to ischemic tissue of at least one active agent comprising a sequence selected from the general formula:

15 R1-Arg-R2-R3-R4-His-Pro-R5, wherein

R1 is Asp or is absent;

R2 is selected from the group consisting of Val, Ala, Ile, Pro, Lys, Norleu, and Leu;

R3 is selected from the group consisting of Ala, Tyr, and Tyr(PO₃)₂; and

20 R4 is selected from the group consisting of Val, Ala, Ile, Norleu, and Leu;

R5 is Phe, Ile, or is absent; and

wherein the compound is not AII.

10. The method of claim 9 wherein the active agent is selected from the group consisting of SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:24, SEQ ID NO:26, SEQ ID

NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, and SEQ ID NO:42.

11. The method of claim 9, further comprising administration of an amount effective for increasing blood flow to ischemic tissue of at least one compound selected from the group consisting of AII, acidic fibroblast growth factor, basic fibroblast growth factor, transforming growth factor alpha, transforming growth factor beta, tumor necrosis factor, platelet-derived growth factor, vascular endothelial growth factor, angiogenin, and haptoglobin.

12. A kit for increasing blood flow to ischemic tissue, comprising:

10 (a) an amount effective for increasing blood flow to ischemic tissue of at least one active agent comprising a sequence selected from the general formula

R1-Arg-R2-R3-R4-His-Pro-R5, wherein

R1 is Asp or is absent;

15 R2 is selected from the group consisting of Val, Ala, Ile, Pro, Lys, Norleu, and Leu;

R3 is selected from the group consisting of Ala, Tyr, and Tyr(PO₃)₂; and

R4 is selected from the group consisting of Val, Ala, Ile, Norleu, and Leu;

R5 is Phe, Ile, or is absent;

wherein the compound is not AII; and

20 (b) instructions for using the amount effective of active agent for increasing blood flow to ischemic tissue.

13. The kit of claim 12 wherein the active agent is selected from the group consisting of SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:31,

SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, and SEQ ID NO:42.

14. The kit of claim 12, further comprising a means for delivery of the active agent.
15. The kit of claim 12, further comprising an amount effective for increasing blood
5 flow to ischemic tissue of at least one compound selected from the group consisting of AII, acidic fibroblast growth factor, basic fibroblast growth factor, transforming growth factor alpha, transforming growth factor beta, tumor necrosis factor, platelet-derived growth factor, vascular endothelial growth factor, angiogenin, and haptoglobin.

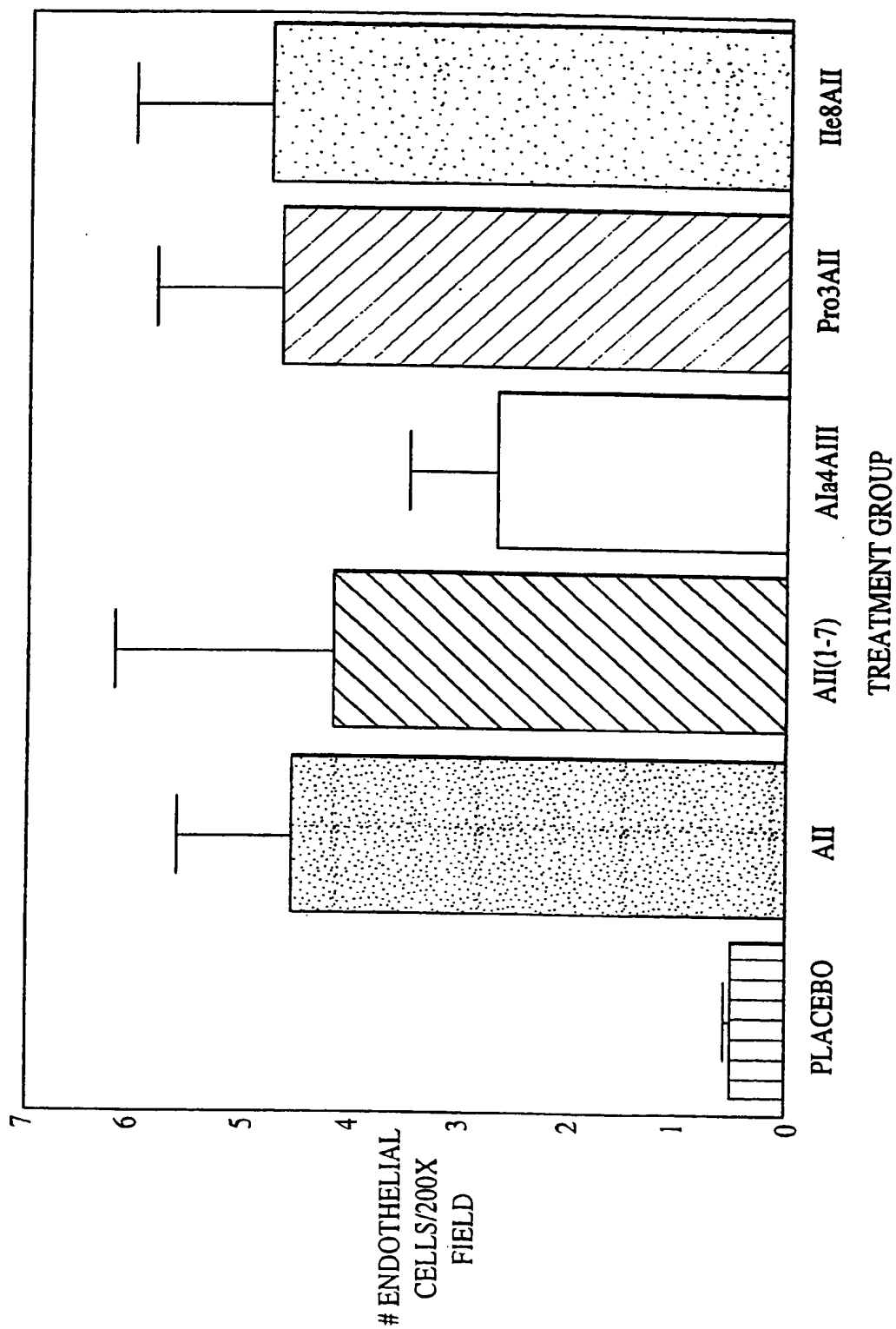


FIG. 1

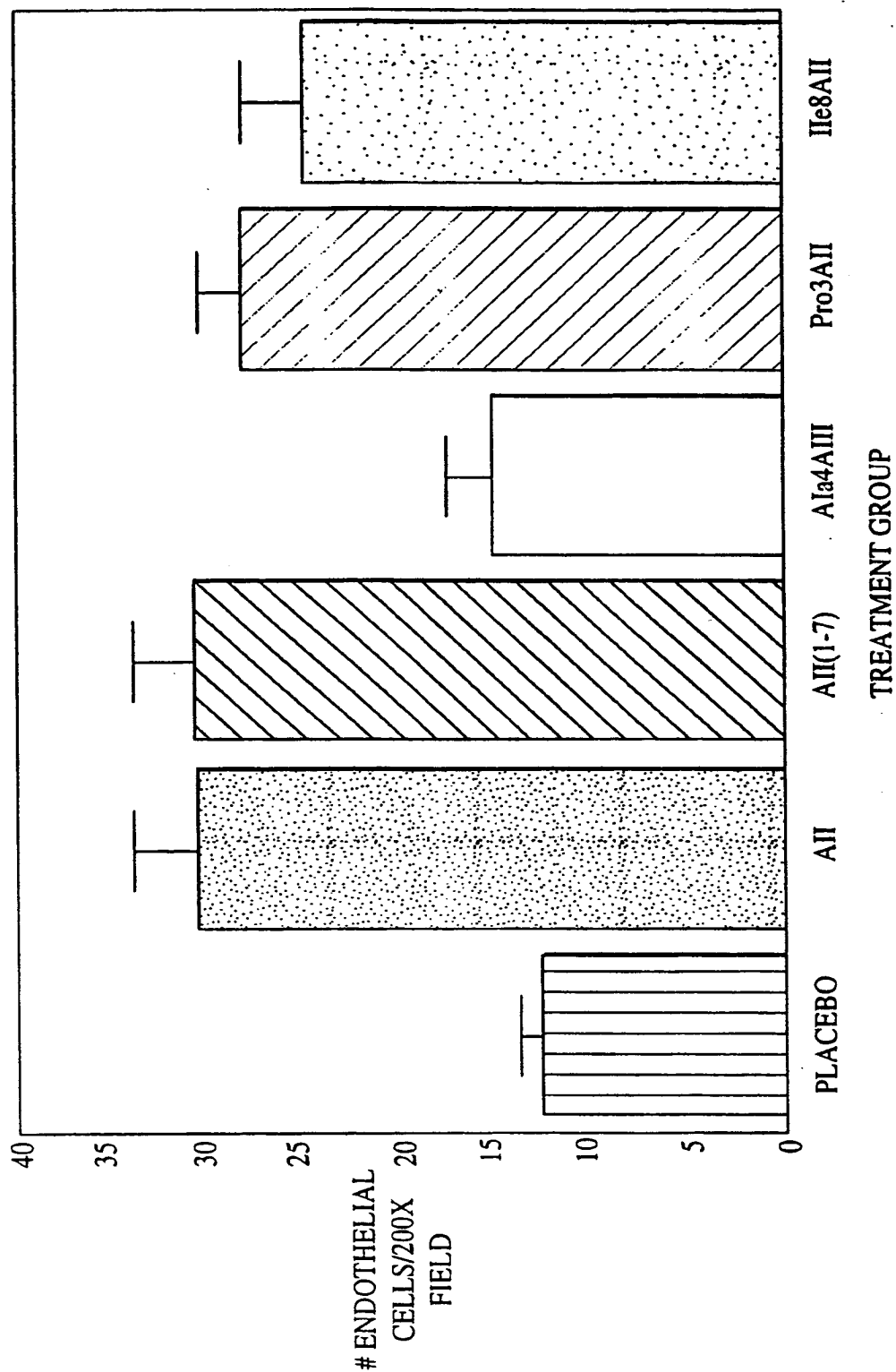


FIG. 2

3/3

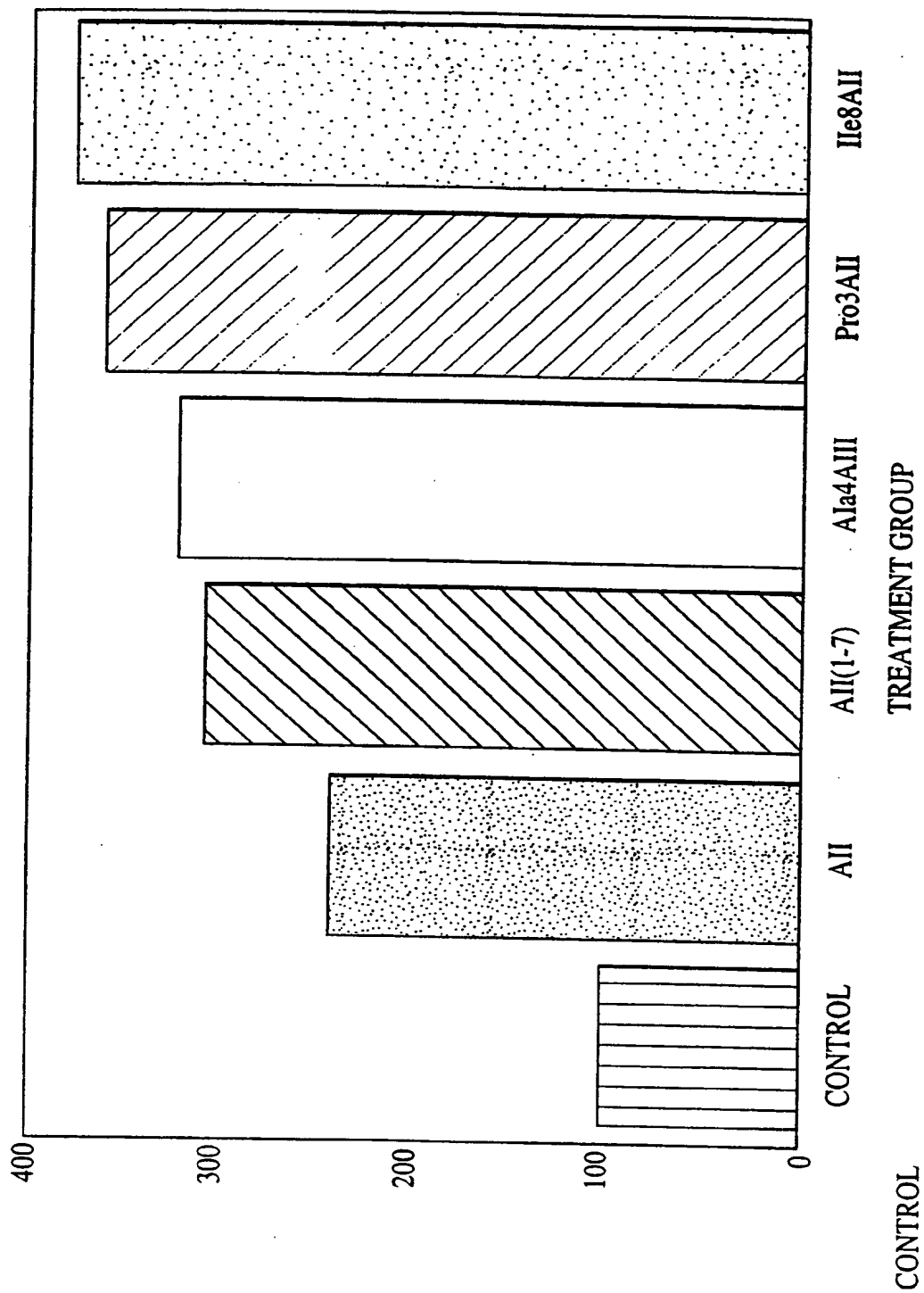


FIG. 3

SEQUENCE LISTING

<110> Rodgers, Kathleen
diZerega, Gere

<120> Methods to Increase Blood Flow to Ischemic Tissue

<130> 98364B

<140> To be assigned

<141> 1999-08-13

<160> 42

<170> PatentIn Ver. 2.0

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1 5

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<400> 9
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1 5

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1

5

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<211> 3
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<210> 15
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class

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<220>
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Gly, Pro, Aib, Acp, or Tyr

<220>

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5

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<210> 37

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<213> Artificial Sequence

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<223> Description of Artificial Sequence:angiotensin I

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10

<210> 38

<211> 7

<212> PRT

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5

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<211> 7

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1 5

<210> 40

<211> 7

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1 5

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<212> PRT

<213> Artificial Sequence

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<400> 42

Asp Arg Val Tyr Ile His Pro Ile
1 5

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 99/18374

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K38/08 C07K7/14 C12N5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 33813 A (DIZEREGA GERE ; RODGERS KATHLEEN E (US); UNIV SOUTHERN CALIFORNIA) 6 August 1998 (1998-08-06) page 2 abstract	1-15
X	WO 96 14858 A (UNIV SOUTHERN CALIFORNIA) 23 May 1996 (1996-05-23) page 1, paragraph 3 page 8, line 1 - line 20	1-15
X	WO 95 08337 A (UNIV SOUTHERN CALIFORNIA) 30 March 1995 (1995-03-30) the whole document	1-15
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

16 December 1999

Date of mailing of the international search report

29/12/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
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Fax: (+31-70) 340-3016

Authorized officer

Cervigni, S

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/18374

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 00492 A (UNIV WASHINGTON ; HARDING JOSEPH W (US); WRIGHT JOHN W (US)) 6 January 1994 (1994-01-06) the whole document claim 21	1-15
A	----- SZEKANECZ Z ET AL: "'Angiotensin and its mediators. Their pathogenic and clinical significanc in "angiogenic diseases" (see comments)!. Az angiogenesis es mediatorai. Patogenetikai es klinikai jelentoseguk "angiogen betegsegekben". ORVOSI HETILAP, (1998 FEB 1) 139 (5) 227-34. REF: 48 , XP002125894 abstract -----	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/18374

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-4, 9-11 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☒ Claims Nos.: 1,5
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1,5

The scope of claims 1 and 5 is unduly broad and speculative. A peptide comprising at least three amino acids out of a formula consisting virtually of all variables cannot be considered to be a clear and concise definition of patentable subject-matter. (Art. 6 PCT). Furthermore, the available experimental data actually only comprise a very small part of the compounds claimed, therefore the claims are also not adequately supported by the description.

Therefore, a meaningful and economically feasible search could not encompass the complete subject-matter of the claims. Consequently, the search has been limited to angiotensin I, II and (closely) related analogues, that is those encompassed by claims 2 and 12. (Art. 17(2)(a)(ii) PCT).

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/18374

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9833813 A	06-08-1998	AU 6650098 A	25-08-1998
WO 9614858 A	23-05-1996	US 5629292 A	13-05-1997
		US 5955430 A	21-09-1999
		AU 4364896 A	06-06-1996
		CA 2205092 A	23-05-1996
		CN 1172434 A	04-02-1998
		EP 0792158 A	03-09-1999
WO 9508337 A	30-03-1995	AU 706333 B	17-06-1999
		AU 7730294 A	10-04-1995
		CA 2172493 A	30-03-1995
		EP 0730465 A	11-09-1996
		JP 9502977 T	25-03-1997
		US 5629292 A	13-05-1997
		US 5955430 A	21-09-1999
		ZA 9407338 A	10-05-1995
WO 9400492 A	06-01-1994	AU 4649293 A	24-01-1994
		CA 2139105 A	06-01-1994
		EP 0647239 A	12-04-1995
		ZA 9304536 A	03-02-1994

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